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at least one of the endogenous nucleotide sequences is inactivated by mutation or deletion.

REMARKS

I. Status of the Application:

With entry of this Amendment, claims 1-51 and 53-69 are pending in the application. Claims 58-69 have been allowed. Office action, page 9. Applicants note the Office has determined that claims 12, 15-28, 40, and 43-69 are free of the prior art. *Id.*

Applicants acknowledge withdrawal of the following rejections:

1. Claims 29-42, 44, 52, and 58-69 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite;

2. Claims 1-11, 12, 14, 29-39, 41, and 42 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Fields in view of any one of Erickson, Korsmeyer or Gallatin, and further in view of Wood; and

3. Claims 1-28 provisionally rejected under the doctrine of obviousness-type double patenting as allegedly unpatentable over claims 49-136 copending application no. 09/305,483. *Id*, page 3.

The Office has objected to claim 57, should claim 52 be found allowable, under 37 C.F.R. § 1.75 because the claims are allegedly substantial duplicates. *Id.*, page 2. Applicants have canceled claim 52. Thus, they request reconsideration and withdrawal of this objection.

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Applicants have amended claims 1, 15, 16, 29, 43, and 44 solely to more clearly recite their invention. Support for these amendments is found in the specification and claims as originally filed. No new matter has been entered.

According to the Office, the Information Disclosure Statement filed July 28, 2000, fails to comply with 37 C.F.R. § 1.98(a)(2) because nine documents could not be located in the file of this application or the parent application files. Office action, page 2. Applicants will re-submit courtesy copies of these documents to the Office.

II. The Claims Comply With 35 U.S.C. § 112, Second Paragraph

Claims 15-57 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter Applicants regard as their invention. Office action, page 3. The Office specifically addresses language appearing in claims 15, 16, 29, 43, 44, and 52. Applicants traverse the rejections.

The Office contends that "[c]laim 15 is vague and indefinite because it is unclear what selected phenotype based on the teachings in the specification is prevented by expression of the luciferase gene." *Id.*, page 4. Applicants have amended claim 15 to delete reference to the selected phenotype.

For claims 16 and 44, the Office contends they are vague and indefinite by reciting "at least one of the endogenous nucleotide sequences is inactivated by reconstitution . . ." and questions whether Applicants should replace "reconstitution" with "mutation." *Id.* Applicants have amended claims 16 and 44 as the Office has suggested.

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The Office contends that in claim 29 there is no antecedent basis for "the selected phenotype" in step (ii), line 2. *Id.* Applicants have amended this claim to recite "expression of the luciferase gene" in place of the language the Office objects to.

With regard to claim 43, the Office asserts that the language "a first heterologous fusion protein" in step (ii) a) and "a second heterologous fusion protein" in step (ii) b) renders the claim vague and ambiguous. To remove the ambiguity, Applicants have amended claim 43 to recite "the first heterologous fusion protein" in step (ii) a) and "the second heterologous fusion protein" in step (ii) b).

The Office also contends that claim 52 is vague and indefinite. Applicants submit that this rejection is moot in light of their cancellation of this claim.

In view of these amendments and remarks, Applicants request reconsideration and withdrawal of the rejections under section 112, second paragraph.

III. The Claims Are Patentable Over the Cited References

A. Bachovin Does Not Anticipate Claims 1, 4, 6, 7, and 14

The Office has rejected claims 1, 4, 6, 7, and 14 as allegedly unpatentable under 35 U.S.C. § 102(e) as anticipated by U.S. Patent No. 5,580,979 to Bachovin. Office action, page 5. Applicants traverse the rejection.

Bachovin is cited as teaching "the yeast two hybrid assay according to Fields et al. and disclose that luciferase can be used in the two hybrid assay as a reporter gene." *Id.*, pages 5 and 6. The Office also contends that Bachovin teaches a peptide binding pair wherein one member of the pair comprises an SH2 domain that recognizes, for example, growth-factor mediated signals, including PDGF. Finally, according to the

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Office, Bachovin teaches the use of the amino-terminal domain of Gal4 as a DNA-binding domain and its carboxy-terminus as a transcriptional activation domain. *Id.*

Applicants respectfully submit that Bachovin does not anticipate (or render obvious) the rejected claims. The rejected claims recite "a peptide binding pair that bind through extracellular interaction in their natural environment . . . ." This aspect of the claimed invention is not taught or suggested by Bachovin.

The Office has cited Bachovin as teaching the use of two-hybrid assays to identify compounds capable of "inhibiting the ability of a particular SH2 domain to bind to a phosphotyrosine-containing polypeptide." Bachovin, col. 20, line 60 to col. 21, line 1. Certain phosphotyrosine-containing polypeptides, such as receptor tyrosine kinases, may span the cell membrane. SH2, that is, Src homology 2, domains, however, are intracellular proteins that are tethered to the interior side of the cell membrane. They do not have an extracellular domain. Therefore, they cannot serve as part of "a peptide binding pair that bind through extracellular interaction in their natural environment . . . ."

The intracellular occurrence of SH2 domains is shown by the attached exhibits. Bachovin identifies several proteins, including Ras GAP, as proteins that contain SH2 domains. Bachovin, col. 2, lines 38-44. Pawson *et al.* teaches that SH2 domains "organize the localization, communication and functional activities of intracellular signaling proteins." Exh. 1, page 504, first column. Enzymes containing an SH2 domain, such as Ras GAP, are described as "constitutively tethered to the membrane through N-terminal myristate and palmitate groups . . . ." *Id.*, page 507, first column. The intracellular location of Ras GAP is confirmed by Vojtek *et al.*, which shows Ras GAP bound to the internal surface of the cell membrane. Exh. 2, page 19926, Fig. 1.

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The teaching of Bachovin is limited to a yeast two-hybrid assay that identifies compounds capable of interfering with the interaction of an SH2 domain and a phosphotyrosine-containing polypeptide. The described SH2 domains and phosphotyrosine-containing polypeptides do not comprise "a peptide binding pair that bind through extracellular interaction in their natural environment." Bachovin, therefore, does not teach (or suggest) all of the limitations of the rejected claims. Accordingly, Applicants request the Office to reconsider and withdraw the rejection.

In addition, Applicants note the Office admits that Bachovin does not teach expressing at least one heterologous fusion protein from an autonomously-replicating plasmid. Office action, page 6. Claims 6 and 7 depend from claim 5, which recites that "at least one of the heterologous fusion proteins is expressed from an autonomously-replicating plasmid." Claim 5 is not rejected as allegedly anticipated by Bachovin. Thus, claims 6 and 7 cannot be anticipated by Bachovin. For this additional reason, the Office should withdraw the rejection of claims 6 and 7.

B. <u>The Claims Would Not Have Been Obvious Over the Prior Art</u>

1. <u>Bachovin in View of Fields and Wood</u>

Claims 1-8, 10, 11, 13, 14, 29-36, 38, 39, 41, and 42 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Bachovin in view of U.S. Patent No. 5,283,173 to Fields *et al.* ("Fields") and U.S. Patent No. 5,641,641 to Wood. Office action, page 6. Applicants traverse.

The Office's interpretation of Bachovin has been discussed. The Office admits that "Bachovin does not teach inactivation of an endogenous sequence; a peptide pair comprising a ligand and a receptor to which the ligand binds; expressing at least one

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heterologous fusion protein from an autonomously-replicating plasmid; any specific yeast strains'; or the two hybrid assay for testing a sample for binding to one or the other of the peptides of the peptide pair." *Id.* These missing aspects of the invention are allegedly taught by Fields, while Wood is cited as providing detailed guidance on the use of luciferase as a reporter gene in yeast. *Id.*, pages 6 and 7.

Based on the alleged teaching in the art, the Office concludes that "[i]t would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute luciferase as a reporter gene into the yeast two hybrid assay of Fields et al, motivated by the teaching of Bachovin that luciferase can be used in the two hybrid assay and further motivated by Wood who teaches the ease with which luciferase can be assayed." *Id.*, page 7. According to the Office, "[o]ne of ordinary skill in the art would reasonably expect that luciferase would function as a reporter gene in the yeast two hybrid assay based on the teachings of Bachovin which indicates the equivalence of different reporter genes in the two hybrid assay." *Id.*

Applicants traverse because the claims are not *prima facie* obvious over the cited references. Claims 1-8, 10, 11, 13, and 14 are directed to a yeast cell comprising nucleotide sequences encoding fusions of first and second peptides "of a known peptide binding pair that bind through extracellular interaction in their natural environment." This aspect of the invention is not taught or suggested by the combination of references cited by the Office.

Applicants have discussed the teaching of Bachovin, which is limited to a yeast two-hybrid assay that identifies compounds capable of interfering with the interaction of an SH2 domain and a phosphotyrosine-containing polypeptide. The described SH2

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domains and phosphotyrosine-containing polypeptides are intracellular proteins. There is no teaching or suggestion in Bachovin of a yeast cell that expresses peptides of a known peptide binding pair that bind through extracellular interaction in their natural environment. On the contrary, the pair of peptides described by Bachovin bind through intracellular interactions in their natural environment.

Fields also discloses a two-hybrid system. Unlike the method of Bachovin, wherein compounds that are capable of affecting the known interaction between two intracellular proteins (SH2 domains and phosphotyrosine-containing polypeptides) are screened for, Fields' method is used to screen for a second, unknown, protein that interacts with a first, known, protein. Fields, col. 3, lines 56-58.

Fields describes a genetic screen to find genes, as yet unidentified, encoding a protein that interacts with a known peptide. Thus, the yeast cells Fields describes do not comprise peptides "of a known peptide binding pair that bind through extracellular interaction in their natural environment." There is no teaching or suggestion in Fields to use a two-hybrid based system to identify compounds that affect the interaction between two known members of a peptide binding pair. This teaching is absent from Fields because Fields' method is used as a screen to identify the second peptide of a peptide binding pair. There is no teaching or suggestion in Fields of taking the next step—using a two-hybrid-based method to identify compounds that interfere with the interaction between the newly identified peptide binding pair. Nor does Fields describe the yeast cells that might be used in such a method.

The combination of Bachovin and Fields does not provide or suggest this aspect of the invention. Adding the teaching of Wood to the combination does not cure this

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deficiency. Wood does not teach anything about two-hybrid assays or the yeast cells used therein. Accordingly, the combination of references does not teach or suggest all of the limitations of claims 1-8, 10, 11, 13, and 14. For this reason, the Office should withdraw the rejection.

Moreover, one of ordinary skill in the art would not have been motivated to combine the teachings of the references as the Office contends. Despite the different aims of Bachovin and Fields, the Office urges that one of ordinary skill in the art would have been motivated "to substitute luciferase as a reporter gene into the yeast two hybrid assay of Fields et al, motivated by the teaching of Bachovin that luciferase can be used in the two hybrid assay and further motivated by Wood who teaches the ease with which luciferase can be assayed." Office action, page 7. This statement, however, is conclusory. To establish a *prima facie* case of obviousness, the Examiner must identify, either in the references themselves or in the knowledge of one of ordinary skill in the art, a teaching, suggestion or motivation to combine the references. That burden has not been met. The fact that the references can be combined does not render the resulting combination obvious unless the prior art also suggests the desirability of the combination. M.P.E.P. § 2143.01.

Bachovin is directed to identifying compounds that interfere with the binding of a pair of known intracellular proteins. Fields, which is directed to a method for identifying an unknown member of a peptide binding pair, makes a single reference to extracellular proteins: "The system can be of value in the identification of new genes. For example, receptors on the cell surface may be identified for known growth factors, toxins, or surface antigens." (Fields, column 3, lines 57-60.) How can Bachovin suggest that it

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would be desirable to modify Fields to provide yeast cells useful in a method of screening for test compounds that interfere with binding between peptides known to interact via extracellular domains when Bachovin does not even teach or suggest a yeast two-hybrid assay utilizing two proteins that interact extracellularly in their natural environment?

In similar circumstances, the Federal Circuit has reversed a rejection under section 103 because the art relied on by the board did not teach or suggest the invention recited in the rejected claims. *In re Fine*, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988). In doing so, the court held that "[t]he Board points to nothing in the cited references, either alone or in combination, suggesting or teaching Fine's invention." *Id.* at 1599. Moreover, the court also found the rejection improper because the primary reference, which focused on the problem of detecting sulfur compounds, contained no suggestion as to how that teaching could be used to solve a different problem, detecting nitrogen compounds. Given the different teaching of the references, the court held that the board erred in affirming the examiner's conclusion that it would have been obvious to substitute the secondary reference's nitrogen detector for the primary reference's sulfur detector:

> The Eads and Warnick references disclose, at most, that one skilled in the art might find it obvious to try the claimed invention. But whether a particular combination might be "obvious to try" is not a legitimate test of patentability.
>
> . . .
>
> Obviousness is tested by "what the combined teachings of the references would have suggested to those of ordinary skill in the art." But it "cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion supporting the combination." And "teachings of references can be combined *only* if there is some suggestion or incentive to do so."

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Id. (Citations omitted.)

Like the references inappropriately combined in *Fine*, there is no teaching or suggestion in Bachovin that its teaching could be used with known extracellular proteins. Bachovin is limited to identifying compounds that interfere with the interaction between two known intracellular binding pairs. There is no suggestion in this reference to use a two-hybrid system to identify compounds that interact via extracellular domains, or the yeast cells used in such a system.

Likewise, Fields is inconsistent with the claimed invention. Fields offers no teaching regarding yeast cells or their use in a two-hybrid system to identify compounds that interfere with the binding between members of a binding pair. Instead, Fields is directed to identifying the binding pair itself. The isolated disclosure in Fields that his method might be used to identify new genes, such as cell surface receptors, is not a suggestion or incentive to modify Bachovin to screen for compounds that disrupt extracellular protein-protein interactions. The rejection relies on hindsight. "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention." *Fine*, 5 U.S.P.Q.2d at 1600. One of ordinary skill in the art would not have been motivated as the Office suggests to render the claimed invention obvious.

Adding the teaching of Wood does not cure the problems evident in the combination of Bachovin and Fields because Wood does not teach or suggest two-hybrid assays. Applicants dispute the Office's characterization of the teaching of Wood. Applicants have argued that Wood describes the use of luciferase as a reporter in assays involving extracts prepared from yeast cells, citing as evidence Wood's

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description at col. 8, line 61 to col. 9, line 46, of the types of sample that can be assayed for the presence of luciferase, and to Examples 1 and 2. In reply, the Office asserts, citing col. 5, lines 32-38 and col. 10, lines 57-64, that "Wood does clearly encompass the in vivo use of the luciferase gene as a reporter gene," and that Wood "characterizes a preferred kit for assaying luciferase in cells." Office action pages 7 and 8. But when the latter cited language in considered, it is evident that Applicants' interpretation of Wood is correct:

> In a preferred kit for assaying for luciferase in cells, in which the luciferase is expressed, there will also be included a solution (or the components for preparing a solution) useful for lysing the cells while preserving (against the action of various enzymes released during lysis) luciferase that might be in the cells in active form, or a form which can be made active.

Wood, col. 10, lines 57-64. Applicants respectfully submit that the relied on teaching of Wood is entirely consistent with their assertion that Wood only teaches the use of luciferase as a reporter in assays involving extracts prepared from yeast cells.

These arguments also apply to claims 29-36, 38, 39, 41, and 42, which are directed to a method that uses at least one yeast cell comprising nucleotide sequences encoding first and second heterologous fusion proteins "of a known peptide binding pair that bind through extracellular interaction in their natural environment." There is no motivation in the references to combine their teachings to arrive at the claimed method, nor do the combined teachings of the references teach or suggest all of the aspects of the invention.

For these reasons, Applicants submit that the Office has not established a *prima facie* case of obviousness of claims 1-8, 11, 13, 14, 29-36, 38, 39, 41, and 42 over the

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cited references. Accordingly, Applicants request reconsideration and withdrawal of this rejection.

2. Bachovin in View of Fields and Wood, and Further in View of Gallatin and Vojtek

The Office has rejected claims 1-11, 13, 14, 29-39, 41, and 42 under section 103(a) as allegedly being unpatentable over Bachovin in view of Fields and Wood, as applied above, and further in view of U.S. Patent No. 5,837,490 to Gallatin *et al.* ("Gallatin"), and in view of Vojtek *et al.*, Mammalian RAS Interacts Directly with the Serine/Threonine Kinase Raf, Cell, 74:205-214 (1993) ("Vojtek"). Office action, page 8. Applicants traverse the rejection.

Bachovin, Fields, and Wood are applied as described above. Gallatin and Vojtek are cited as teaching the use of LexA in the yeast two hybrid assay. Based on this asserted teaching, the Office concludes that "[i]t would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the DNA binding domain of LexA into the yeast the hybrid assay of fields et al based on the teaching of Gallatin et al as to the equivalence of the LexA DNA binding domain and other DNA binding domains." *Id.*

Applicants respectfully submit that the claims are not *prima facie* obvious for the reasons set forth above. Gallatin and Vojtek are cited as teaching the use of LexA in a yeast two-hybrid assay. This teaching does not cure the deficiencies of the combined teachings of Bachovin, Fields, and Wood, as described above. Therefore, adding the teachings of Gallatin and Vojtek to Bachovin, Fields, and Wood does not establish a *prima facie* case of obviousness. Applicants request the Office to reconsider and withdraw the rejection.

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IV. Rejection of Claims 1-28 for Obviousness-Type Double Patenting

Claims 1-28 stand rejected under the doctrine of obviousness-type double patenting over claims 1-88 of U.S. Patent No. 6,284,519 in view of U.S. Patent No. 5,580,979 to Bachovin . (Office action, page 9.) Applicants traverse this rejection, however, they respectfully request that this issue be held in abeyance until allowable subject matter is indicated.

CONCLUSION

In view of the above amendments and remarks, Applicants submit that this application is in condition for allowance. An early and favorable response from the Office is earnestly solicited.

Please grant any extensions of time required to enter this Amendment and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

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By: _____
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APPENDIX I
Claim Amendments

1. (Amended) A yeast cell comprising:

a) a nucleotide sequence encoding a first heterologous fusion protein comprising a first peptide of a known peptide binding pair that bind through extracellular interaction in their natural environment, or a segment thereof, joined to a transcriptional activation protein DNA binding domain;

b) a nucleotide sequence encoding a second heterologous fusion protein comprising a second peptide of the binding pair, or a segment thereof, joined to a transcriptional activation protein transcriptional activation domain;

wherein binding of the first peptide or segment thereof and the second peptide or segment thereof reconstitutes a transcriptional activation protein; and

c) a luciferase gene activated under positive transcriptional control of the reconstituted transcriptional activation protein.

15. (Amended) A yeast cell comprising:

a) a nucleotide sequence encoding a first heterologous fusion protein comprising a first peptide of a peptide binding pair, or a segment thereof, joined to a transcriptional activation protein DNA binding domain;

b) a nucleotide sequence encoding a second heterologous fusion protein comprising a second peptide of the peptide binding pair, or

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a segment thereof, joined to a transcriptional activation protein transcriptional activation domain;

wherein the nucleotide sequence encoding either the first or second heterologous fusion protein is present in an effective copy number of at least 5 copies per yeast cell and the nucleotide sequence encoding the other heterologous fusion protein is present at a copy number of 1 or 2 per yeast cell;

and

wherein binding of the first peptide or segment thereof and the second peptide or segment thereof reconstitutes a transcriptional activation protein; and

c) a luciferase gene activated under positive transcriptional control of the reconstituted transcriptional activation protein[, wherein expression of the luciferase gene prevents exhibition of a selected phenotype].

16. (Amended) The yeast cell of claim 15 further comprising at least one endogenous nucleotide sequence selected from the group consisting of a nucleotide sequence encoding the transcriptional activation protein DNA binding domain[,] and a nucleotide sequence encoding the transcriptional activation protein transcriptional activation domain, wherein at least one of the endogenous nucleotide sequences is inactivated by [reconstitution] <u>mutation</u> or deletion.

29. (Twice amended) A method of detecting the interaction of a first peptide and a second peptide of a peptide binding pair in the presence of a test sample, comprising:

(i) culturing at least one yeast cell, wherein the yeast cell comprises;

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a) a nucleotide sequence encoding a first heterologous fusion protein comprising the first peptide of a known peptide binding pair that bind through extracellular interaction in their natural environment, or a segment thereof, joined to a transcriptional activation protein DNA binding domain;

b) a nucleotide sequence encoding a second heterologous fusion protein comprising the second peptide, or a segment thereof, joined to a transcriptional activation protein transcriptional activation domain;

wherein binding of the first peptide or segment thereof and the second peptide or segment thereof reconstitutes a transcriptional activation protein; and

c) a luciferase gene activated under positive transcriptional control of the reconstituted transcriptional activation protein;

(ii) incubating the test sample with the yeast cell under conditions suitable to detect [the selected phenotype] expression of the luciferase gene; and

(iii) detecting the interaction of the first peptide and the second peptide by determining the level of expression of the luciferase gene.

43. (Amended) A method for determining whether a test sample interacts with a first or second peptide of a peptide binding pair, comprising:

(i) culturing at least one first yeast cell, wherein the first yeast cell comprises;

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a) a nucleotide sequence encoding a first heterologous fusion protein comprising the first peptide or a segment thereof joined to a transcriptional activation protein DNA binding domain;

b) a nucleotide sequence encoding a second heterologous fusion protein comprising the second peptide or a segment thereof joined to a transcriptional activation protein transcriptional activation domain;

wherein the nucleotide sequence encoding the first heterologous fusion protein is present in an effective copy number of at least 5 copies per yeast cell and the nucleotide sequence encoding the second heterologous fusion protein is present at a copy number of 1 or 2 per yeast cell;

and

wherein binding of the first peptide or segment thereof and the second peptide or segment thereof reconstitutes a transcriptional activation protein; and

c) a luciferase gene activated under positive transcriptional control of the reconstituted transcriptional activation protein;

(ii) culturing at least one second yeast cell, wherein the second yeast cell comprises;

a) a nucleotide sequence encoding [a] the first heterologous fusion protein comprising the first peptide or a segment thereof joined to a transcriptional activation protein DNA binding domain;

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b) a nucleotide sequence encoding [a] the second heterologous fusion protein comprising the second peptide or a segment thereof joined to a transcriptional activation protein transcriptional activation domain;

wherein the nucleotide sequence encoding the second heterologous fusion protein is present in an effective copy number of at least 5 copies per yeast cell and the nucleotide sequence encoding the first heterologous fusion protein is present at a copy number of 1 or 2 per yeast cell;

and

wherein binding of the first peptide or segment thereof and the second peptide or segment thereof reconstitutes a transcriptional activation protein; and

c) a luciferase gene activated under positive transcriptional control of the reconstituted transcriptional activation protein;

(iii) incubating a test sample with the first and second yeast cells under conditions suitable to detect luciferase activity;

(iv) detecting the luciferase activity produced by the first and second yeast cells; and

(v) comparing the detected luciferase activity of the first and second yeast cells, wherein lower luciferase activity in one of the yeast cells compared to the other yeast cell indicates that the test sample binds to the heterogeneous fusion protein encoded by the nucleotide sequence present at a copy number of 1 or 2 in that yeast cell exhibiting lower

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luciferase activity, thereby affecting the binding interaction of the peptide binding pair.

44. (Twice amended) The method of claim 43 wherein either or both of the first and second yeast cells further comprises at least one endogenous nucleotide sequence selected from the group consisting of a nucleotide sequence encoding the transcriptional activation protein DNA binding domain[,] and a nucleotide sequence encoding the transcriptional activation protein transcriptional activation domain, wherein at least one of the endogenous nucleotide sequences is inactivated by [reconstitution] mutation or deletion.

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SH2 domains, interaction modules and cellular wiring

Tony Pawson, Gerald D. Gish and Piers Nash

SH2 domains serve as the prototype for a growing family of protein-interaction modules, characteristic of polypeptides involved in transmitting signals from external and internal cues. The specific interactions of proteins with one another, and with other cellular components such as phospholipids and nucleic acids, provide a very general device to organize cellular behavior. We discuss the idea that rewiring of the cell's interaction network by pathogenic microorganisms and mutant cellular proteins contributes to dysregulation of cell signaling and thus to disease.

Over the past 10 years, the Src-homology 2 (SH2) domain has served as the prototype for a large and growing family of molecular-interaction domains that together organize the localization, communication and functional activities of intracellular signaling proteins. In addition to regulating signaling from many types of cell-surface receptors, interaction domains are important in controlling cellular processes such as protein trafficking and degradation, cell-cycle progression, cell survival, polarity, gene expression and DNA repair. Indeed, the specific interactions of proteins one with another, and with other cellular components such as phospholipids and nucleic acids, provide a very general device to organize cellular function and to direct specific cellular responses to external signals. The reiterated use of interaction domains and binding motifs to control cellular behavior provides an opportunity to comprehensively explore the wiring circuitry of eukaryotic cells. Here, we use the SH2 domain to exemplify some of the common features of interaction domains, focusing on issues of particular interest to our laboratory.

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SH2 domains as a prototype for protein-interaction modules

By 1991, the basic functions of the SH2 domain, and the modular nature of intracellular signaling proteins, were emerging[1]. The SH2 domain was originally defined in the arcane retroviral oncoprotein v-Fps, which has a constitutively active tyrosine kinase domain at its C-terminus. The SH2 domain was identified as a sequence of approximately 100 amino acids, just N-terminal to the catalytic domain, that is not required for kinase activity *per se* but which regulates the function of the kinase domain and its interactions with targets in the host cell[2]. As a consequence, the SH2 domain is crucial for the efficient transforming activity of v-Fps. A related sequence was noted in the Src and Abl tyrosine kinases, leading to the 'homology domain' nomenclature (with SH1 being the kinase domain) and the suggestion that the SH2 domain is a conserved non-catalytic module involved both in modifying kinase activity and in directing tyrosine kinase interactions with cellular substrates.

Ensuing work led to the isolation of additional retroviral and cellular proteins that contain SH2 domains, notably phospholipase C (PLC)-γ1, Ras GTPase-activating protein (RasGAP) and the v-Crk oncoprotein, and the realization that these often contain another small non-catalytic module, the SH3 domain[3–5]. Although these proteins have diverse biochemical functions, in regulating phosphoinositide metabolism or Ras GTPases and in the assembly of protein complexes, respectively, they were apparently all involved in tyrosine kinase signaling. This suggested that SH2 domains might have a unique function in coupling otherwise unrelated polypeptides to receptor tyrosine kinases (RTKs)[6,7]. Indeed, SH2 domains, expressed in isolation as recombinant proteins, bound directly and specifically to autophosphorylated RTKs through their ability to recognize selected phosphotyrosine sites[7–9]. This resolved the puzzling observation that, in a growth-factor-stimulated cell, the principal substrate for tyrosine phosphorylation is often the growth factor receptor itself. Such autophosphorylation was realized to have two functions – one being to potentiate kinase activity[10,11], the other to create docking sites for proteins with SH2 domains, which, following receptor binding, activate downstream signaling pathways.

Analysis of specific phosphopeptide motifs from activated RTKs, as well as the use of degenerate phosphopeptide libraries, showed that, in addition to their common ability to bind to phosphotyrosine, SH2 domains recognize between 3–6 residues C-terminal to the phosphorylated tyrosine in a fashion that differs from one SH2 domain to another[12]. This ability of SH2 domains to discriminate between different phosphorylated motifs provides an element of specificity, such that the sequence context of the autophosphorylation sites of a receptor determine which SH2-containing targets it recruits, and thus which biochemical pathways it activates[13].

It is now evident that a substantial number of protein-interaction domains recognize short, defined peptide motifs within larger polypeptides[14,15] (see www.mshri.on.ca/pawson/research1.html), and numerous domains have been defined in signaling proteins [see the SMART database (http://smart.embl-heidelberg.de/)]. Complex formation is often dependent on posttranslational modification of the peptide ligand, and indeed it appears that a major function of

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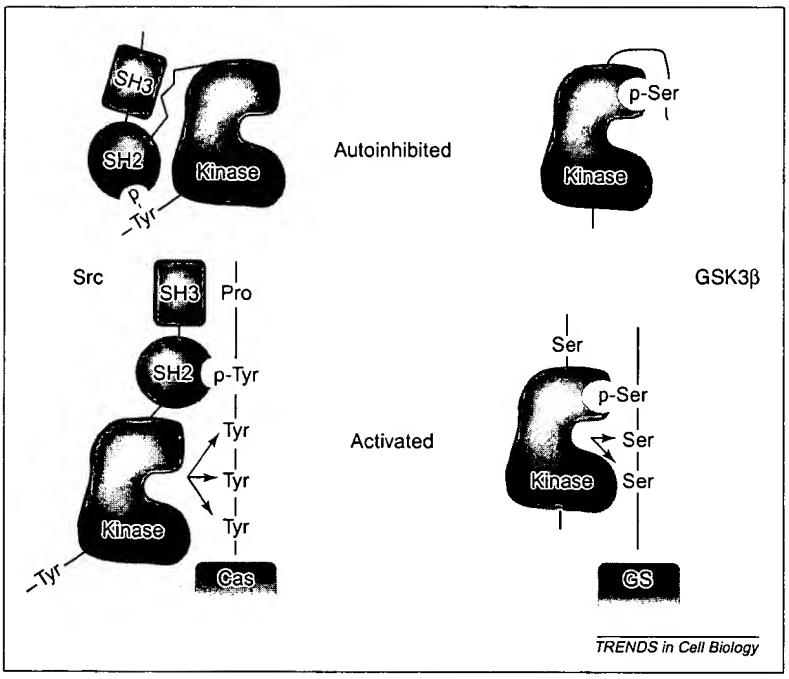

Fig. 1. Similar phospho-dependent mechanisms regulate the activity and substrate recognition of the c-Src tyrosine kinase and the GSK3β serine/threonine kinase. In both enzymes, inhibitory phosphorylation leads to an intramolecular interaction with a substrate docking site (the SH2 domain or phosphoserine-binding pocket) and to autoinhibition. Dephosphorylation of the inhibitory site leads to enzyme activation and release of the substrate-binding sites (SH2/SH3 domains for Src and the phosphoserine-binding pocket for GSK3β), which can engage substrates such as $p130^{cas}$ (Cas) or glycogen synthase (GS) for multi-site phosphorylation. Abbreviations: Pro, proline residue; Ser, serine residue; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain; Tyr, tyrosine residue.

modifications such as phosphorylation is to control protein–protein interactions. Alternatively, the association can be relatively stable, as in the binding of SH3 domains to Pro-X-X-Pro motifs[16]. Knowing the consensus sequences of interaction domains and their peptide ligands makes it possible to predict the binding properties of receptors or intracellular proteins based on their primary sequences (http://cansite.bidmc.harvard.edu/cantley85.phtml). This said, the folded structure of the ligand is often important for recognition by interaction domains, placing some limitations on this predictive approach[17].

Tyrosine phosphorylation therefore serves as a switch to recruit SH2-containing targets. These targets can be activated simply through their binding to the receptor, by juxtaposing them next to membrane-associated substrates. Alternatively receptor-binding might relieve inhibitory constraints on enzymatic activity imposed by intramolecular interactions of the SH2 domain with the catalytic domain. In addition, once bound to the receptor, the SH2-containing protein can itself become a preferred substrate for phosphorylation, resulting either in a conformational change or in the creation of docking sites for additional SH2 proteins. Clearly these are not mutually exclusive modes of regulation.

While the binding of tyrosine kinases to their targets was initially thought to be a peculiarity of this class of protein kinases, it now appears that protein-serine/threonine kinases undergo docking interactions with their substrates and regulators, either directly or through their common association with a scaffolding protein[18,19]. This solves a problem posed by the limited specificity with which the active sites of protein kinases recognize potential phosphorylation sites. For protein-serine/threonine kinases, such as MAP kinases, to phosphorylate a small subset of cellular proteins, they must first recruit their targets through docking interactions and subsequently pick out appropriate phosphorylation sites within the associated substrates.

An example is provided by glycogen synthase kinase 3 (GSK3), which in insulin signaling requires previous phosphorylation of its substrates (i.e. glycogen synthase) on a serine at the +4 position relative to the GSK3 phosphorylation site. The GSK3β kinase domain has a binding pocket for the priming phosphoserine on the substrate, with an arginine poised to interact with the phosphate (a common feature of phospho-dependent interactions)[20]. Intriguingly GSK3β is autoinhibited by phosphorylation at Ser9, which consequently occupies the substrate-recognition pocket (Fig. 1). This scheme is reminiscent of the Src tyrosine kinase, which is also autoinhibited through a phospho-dependent intramolecular interaction (see below). In the Wnt signaling pathway, GSK3β is colocalized with its substrate β-catenin through their common association with a scaffolding protein, axin[21]. Thus docking interactions between enzyme and substrate are a very general feature of protein kinases (and other regulatory enzymes), rather than being an aspect unique to tyrosine kinase signaling.

SH2 and SH3 domains in signaling to growth, survival and cytoskeletal pathways

Unlike SH2 domains, SH3 domains bind to proline-rich sequences independently of posttranslational modifications[22], although serine/threonine phosphorylation of the ligand can block such interactions[23]. Proteins of the Grb2, Nck and Crk families contain an SH2 and multiple SH3 domains but lack intrinsic catalytic function. We termed such proteins adaptors because they can couple a phosphotyrosine signal, recognized by the SH2 domain, to downstream targets with proline-rich motifs that bind to the SH3 domains[1]. Thus, the Grb2 SH2 domain binds to specific pTyr-X-Asn motifs on activated receptors or cytoplasmic docking proteins, while its SH3 domains associate with the Ras GDP–GTP exchange factor (GEF) Sos[24,25]. Genetic data from invertebrates showed that these interactions are not simply a biochemical curiosity but are crucial for RTKs to activate the Ras-MAP kinase pathway *in vivo* and thus for normal development[26–28]. The mammalian Grb2–Sos pathway is also important at various stages of embryonic development and postnatal function and for tumor formation *in vivo*[29,30]. These experiments define a core signaling pathway that is conserved in evolution and has multiple biological functions (Fig. 2). The challenge is to find how this pathway is modulated to yield distinct biological responses in different cells and tissues.

The N-terminal Grb2 SH3 domain is primarily responsible for signaling through Sos[30], while the C-terminal SH3 domain binds to the Gab1/Gab2 docking proteins, facilitating their recruitment to RTKs such as the epidermal growth factor (EGF) receptor[31]. The consequent phosphorylation of Gab1 creates binding sites for additional SH2 proteins, notably the p85

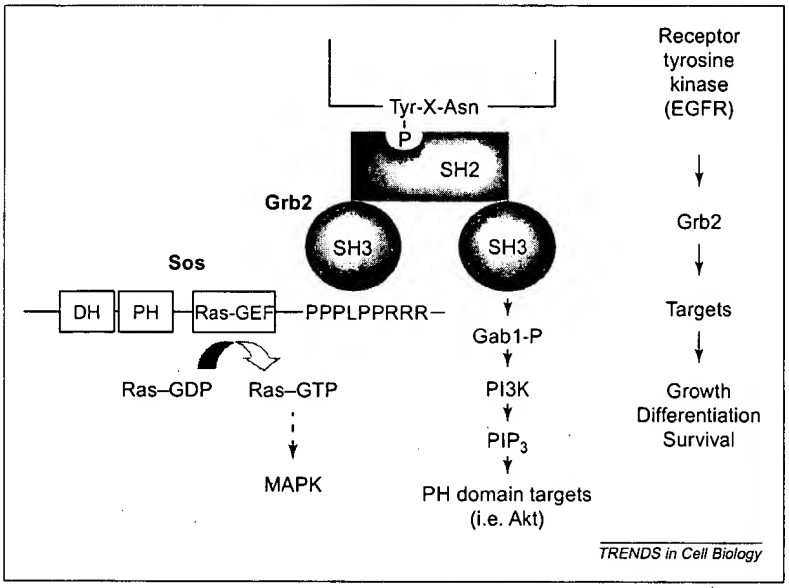

Fig. 2. The Grb2 SH2–SH3 adaptor couples a pTyr-X-Asn docking site to multiple downstream targets through a series of protein–protein and protein–phospholipid interactions. Abbreviations: Asn, asparagine residue; Ras-GEF, Ras GDP–GTP exchange factor catalytic domain; DH, Dbl homology domain; EGFR, epidermal growth factor receptor; PH, pleckstrin homology domain; MAPK, mitogen-activated protein kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain; Tyr, tyrosine residue.

subunit of phosphoinositide 3-kinase (PI3K)[32], resulting in the production of phosphatidylinositol (3,4,5)-trisphosphate [which binds to pleckstrin homology (PH) domains] and the activation of survival pathways through PH-containing proteins such as the Akt and PDK1 protein-serine/threonine kinases[33,34].

Two main points emerge. First, Grb2 coordinates the activation of distinct signaling pathways that promote cell growth, differentiation and survival. Second, the regulation of these pathways involves a succession of protein–protein and protein–phospholipid interactions, such that a complex signaling network is established from the reiterated use of binary molecular interactions. A similar situation pertains for the Nck adaptor, which links specific phosphotyrosine sites to SH3-binding proteins, such as N-WASP, WASP-interacting protein (WIP) and the PAK protein serine/threonine kinase that regulates the actin cytoskeleton[16,35]. Subtle differences in SH2/SH3 domain-binding specificity are therefore used to convey phosphotyrosine signals to quite different cytoplasmic targets.

Binding properties of SH2 domains

Conventional SH2 domains must achieve something of a balancing act. Their affinity for an unphosphorylated site must not be too high, or binding will not be regulated by phosphorylation. At the same time, they must obtain sufficient binding energy from the recognition of adjacent residues to obtain a degree of binding specificity, but have sufficiently high off-rates for rapidly reversible signaling. A structural basis for this specificity has been provided by numerous crystal and solution structures of SH2 domains bound to phosphotyrosine-containing peptides[15]. The SH2 domain fold, comprising a central anti-parallel β-sheet sandwiched between two α-helices (Fig. 3), provides a positively charged pocket on one side of the β-sheet for binding of the phosphotyrosine moiety of the ligand and an extended surface on the other for binding residues C-terminal to the phosphotyrosine. Differences in the

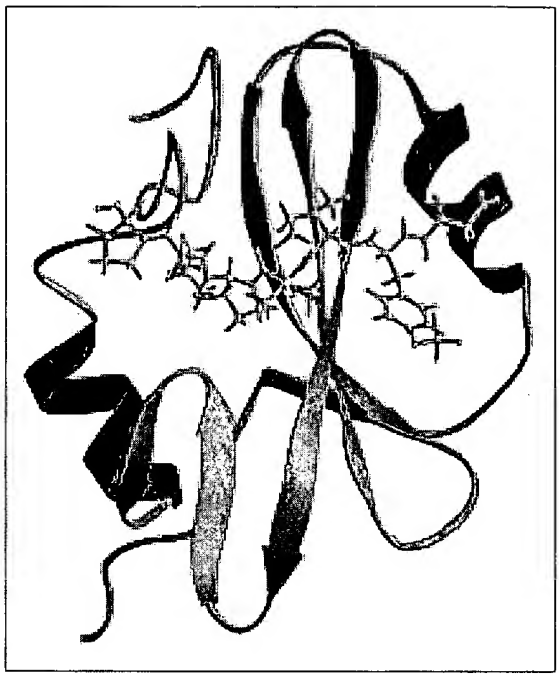

Fig. 3. Secondary structure representation of the phospholipase-Cγ1 C-terminal Src-homology 2 (SH2) domain as bound to the platelet-derived growth factor (PDGF) receptor peptide DNDpYIPLPDPK (gold)[81]. The SH2 domain β-sheets and loops are green, and the α-helices are depicted in blue.

molecular architecture of this extended surface can have significant effects on ligand binding specificity. For example, mutation of a threonine residue in the Src SH2 domain to tryptophan converts ligand binding specificity from the Src-like pTyr-Glu-Glu-Ile to the signature Grb2 binding motif pTyr-X-Asn (Fig. 4)[36]. This mutant Src SH2 domain behaves biologically like a Grb2 SH2 domain, indicating that biological activity correlates with biochemical binding specificity. The binding affinity and specificity of SH2 proteins for phosphorylated partners is markedly increased by linking two SH2 domains in tandem, as occurs in proteins such as the Shp2 tyrosine phosphatase, PLC-γ1 (Refs 37 and 38), and the tandem SH2 domains of the ZAP-70 tyrosine kinase, which bind in an obligatory fashion to bisphosphorylated sites in the signaling subunits of the T-cell antigen receptor[38].

Families of SH2 domain proteins

SH2 domains are embedded in a series of functionally quite different proteins (Fig. 5), which we discuss in turn below.

Enzymes

A number of proteins contain one or two SH2 domains linked to catalytic sequences, such as protein/lipid kinase or phosphatase domains, phospholipase C, a RasGAP domain or a Rho-family GEF domain. Such proteins often contain other interaction modules, commonly SH3 and PH domains, which together with the SH2 domain can regulate the activity of the linked catalytic domain and juxtapose the enzyme with its substrates[14]. In the case of autoinhibited Src-family

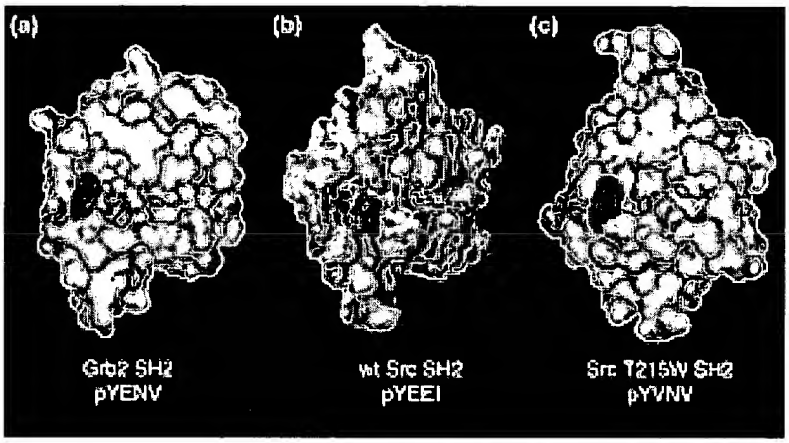

Fig. 4. Structural analysis provides a basis for understanding Src-homology 2 (SH2) domain binding specificity. Crystal structures of the Grb2 (a) and Src SH2 (b) domains bound to optimal phosphopeptides illustrate the different modes of ligand binding. A switch in the ligand preference of the Src SH2 domain to pTyr-Val-Asn-Val is achieved through mutation of a threonine residue, highlighted in blue on the surface of the Src SH2, to the tryptophan shown as red in the Src T215W SH2 domain (c). In a similar fashion, the molecular architecture of the Grb2 SH2 domain uses a tryptophan (red) to define its binding to pTyr-X-Asn ligands.

kinases, the SH2 domain binds to a C-terminal phosphotyrosine site, positioning the SH3 domain to recognize the linker sequence between the SH2 and kinase domains (Fig. 1)[39]. The intramolecular binding of the c-Src SH2 and SH3 domains results both in repression of kinase activity, and in the sequestration of the interaction domains away from exogenous binding partners. Molecular dynamics simulations of autoinhibited c-Src suggest that its SH2 and SH3 domains are tightly coupled through a short connecting sequence, creating a cooperative mechanism for inhibiting kinase activation[40]. c-Src activation can be achieved either by dephosphorylation of the C-terminal inhibitory phosphotyrosine site (or in oncogenic variants by loss of the C-terminal tail) or by binding of high-affinity ligands to the SH2 or SH3 domains (Fig. 1). The consequent unraveling of the autoinhibited structure results in activation of the kinase domain, potentially aided by decoupling of the SH3 and SH2 domains, and binding of the SH2 and SH3 domains to cellular proteins, thereby targeting the kinase domain to its substrates[41,42].

Src-family kinases are constitutively tethered to the membrane through N-terminal myristate and palmitate groups; however, members of the Tec family of cytoplasmic tyrosine kinases, such as the B-cell-specific Btk, have an N-terminal PH domain that binds selectively to phosphatidylinositol (3,4,5)-trisphosphate[43]. Btk activation therefore requires the prior stimulation of PI3K to generate phosphatidylinositol (3,4,5)-trisphosphate and thereby to recruit Btk to the plasma membrane. These interactions are important as mutations that inactivate the Btk PH or SH2 domains cause a B-cell immunodeficiency (X-linked agammaglobulinemia), as does loss of Btk kinase activity[44].

The Shp2 tyrosine phosphatase contains tandem SH2 domains. In the repressed state, a loop in the N-terminal SH2 domain inserts into the phosphatase active site, engaging the catalytic cysteine and promoting an inactive conformation[45]. *In vitro*, the phosphatase can be activated about 100-fold by bisphosphorylated peptides, which bind to the SH2 domains and liberate an active phosphatase domain. *In vivo*, activated RTKs create docking sites for the Shp2 SH2 domains, simultaneously stimulating Shp2 phosphatase activity and potentially localizing the enzyme close to its substrates.

Oncogenic proteins and pathogenic interactions

SH2 domains were originally identified in the context of retroviral transforming proteins. Protein–protein interactions also appear crucial for the transforming activity of tyrosine kinases encoded by cellular oncogenes. An example is the human Bcr–Abl oncoprotein, responsible for chronic myelogenous leukemia[46], in which the Bcr sequence induces oligomerization, stimulating Bcr–Abl autophosphorylation and activation[47]. Both the kinase and SH2 domains are important for Bcr–Abl transformation, and inhibition of kinase activity by the drug STI-571/Glivec has a striking therapeutic effect in CML patients[48]. Interestingly, Tyr177 within the Bcr region of Bcr–Abl becomes phosphorylated, and binds the Grb2 SH2 domain, potentiating Bcr-Abl malignancy[49]. Furthermore, the C-terminal tail of Abl has a number of binding motifs, including proline-rich sequences that recruit SH2/SH3 adaptors such as Crkl. Thus the Bcr–Abl oncoprotein is a modular polypeptide that is activated as a consequence of inappropriate protein–protein interactions, resulting in aberrant signaling through its kinase and interaction domains.

Oncoproteins such as Bcr–Abl rewire cell signaling by driving pathological molecular interactions. A number of proteins from pathogenic bacteria and viruses also recruit host proteins and thereby redirect cellular behavior. For example, the latent membrane protein (LMP) 2A protein of Epstein–Barr virus (EBV), which is expressed in latently infected B cells, has tyrosine-based motifs that become phosphorylated and recruit the SH2 domains of the B-cell tyrosine kinases Lyn and Syk[50]. In addition, LMP2A has two PPPPY motifs that bind to the WW interaction domains of Hect-family E3 protein–ubiquitin ligases (i.e. AIP4/Itch), which in turn can ubiquitinate Lyn, which is consequently degraded[51,52]. The ability of LMP2A to recruit these B-cell signaling proteins might enable latently infected B cells to survive, while suppressing signaling through the B-cell antigen receptor, which would otherwise activate EBV replication. In a similar vein, the Tir protein of enteropathogenic *Escherichia coli* reorganizes the host cell actin cytoskeleton in part through the ability of Tir to recruit the Nck SH2/SH3 adaptor and the associated N-WASP protein[53]. Thus bacterial and viral proteins, by acquiring short recognition motifs, can manipulate the cellular interaction network to meet the needs of the pathogen.

Docking proteins

Many SH2 proteins nucleate the formation of multiprotein complexes. Mammalian ShcA has a C-terminal SH2 domain and an N-terminal phosphotyrosine-binding (PTB) domain, which binds to Asn-Pro-X-pTyr motifs[17]. Although both the SH2 and PTB domains recognize phosphotyrosine motifs

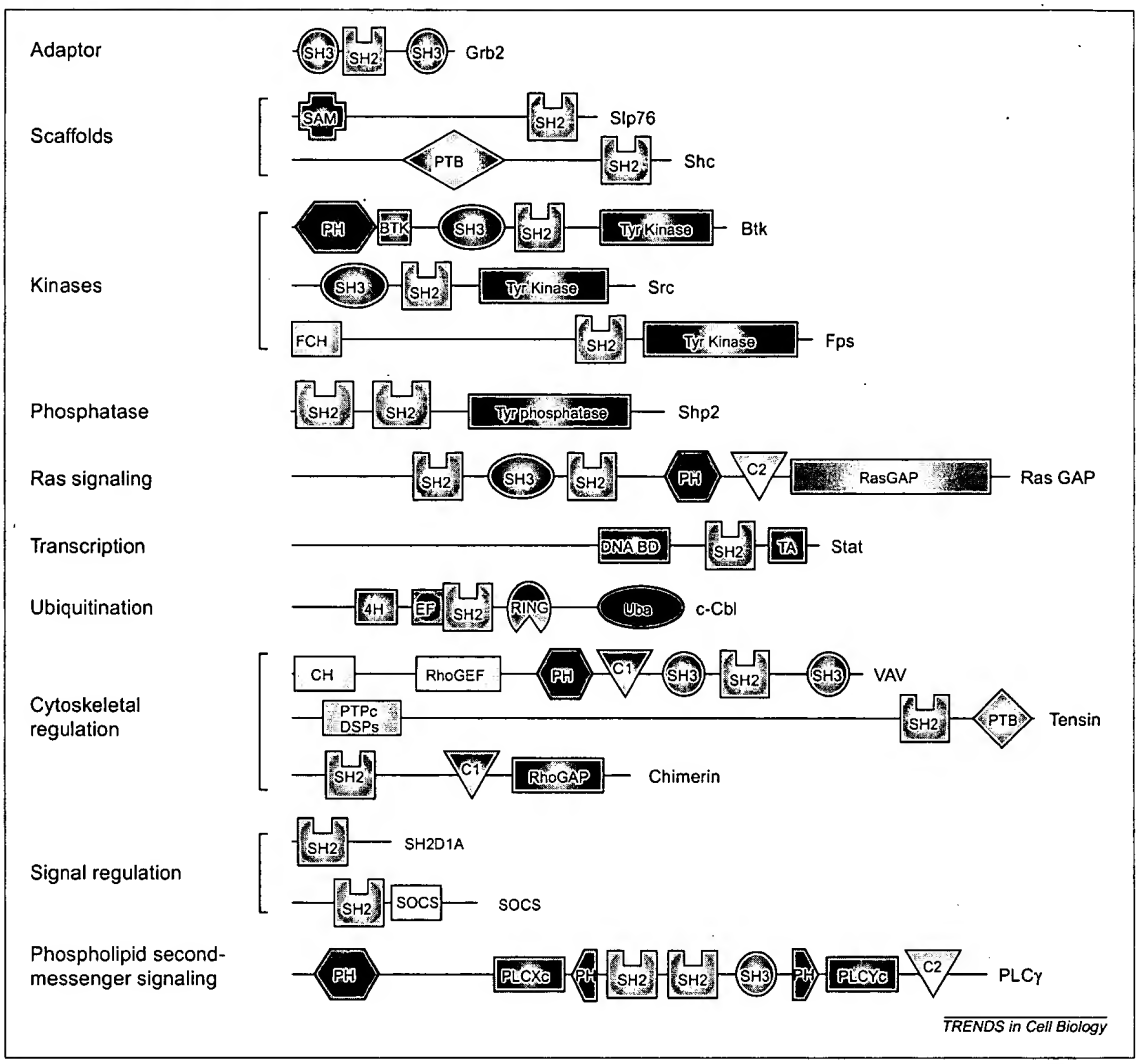

Fig. 5. Modular nature of signaling proteins. Representative members from various SH2 domain families and the positional organization of the domain modules within these proteins are illustrated. See the text for further details. Additional information on individual domains can be found at www.mshri.on.ca/pawson/research1.html and http://smart.embl-heidelberg.de Abbreviations: CH, calponin homology; C1, PKC conserved region 1 phorbol ester binding; C2, PKC conserved region 2 calcium-dependent phospholipid binding; DNA BD, DNA-binding domain; EF, EF-hand; FCH, Fes/CIP4 homology domain; 4H, four-helix bundle; GAP, GTPase-activating domain; GEF, GDP–GTP exchange factor; PH, pleckstrin homology domain; PLC, phospholipase C; PTB, phosphotyrosine-binding domain; PTPc/DSPc, protein tyrosine phosphatase, unknown specificity; SAM, sterile alpha motif; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain; SOCS, suppressor of cytokine signaling; STAT signal transducer and activator of transcription; TA, transcriptional activation domain; Uba, ubiquitin-associated domain.

on activated receptors, they are structurally distinct and recognize phosphopeptides in quite different fashions. The ShcA PTB and SH2 domains flank a central region with two principal sites of tyrosine phosphorylation, both in Tyr-X-Asn sequences. Once phosphorylated, ShcA can recruit multiple Grb2 molecules and potentially other SH2-domain proteins[14]. Thus, ShcA functions, in part, as an auxiliary docking subunit of activated RTKs, which can extend or amplify the binding potential of a receptor. A number of such docking proteins have been identified, including IRS-1 and FRS2; typically these have a means of membrane attachment (such as a PH domain), a PTB domain to bind to activated RTKs and multiple tyrosine sites that, upon phosphorylation, recruit SH2 proteins[54,55].

Adaptors

Adaptor proteins, as discussed above, often comprise a single SH2 domain and multiple SH3 domains, which convert a specific phosphotyrosine input to a defined signaling output. One might wonder why the cell makes use of adaptors, rather than simply fusing an SH2 domain to the relevant enzymatic target. For example, the early functions of Grb2 in mammalian embryonic development can be supported by a variant of Sos carrying the Grb2 SH2 domain at its C-terminus[30]. One possibility is that adaptors provide increased opportunities for positive and negative regulation; in addition, a single adaptor can potentially recruit multiple SH3-binding proteins involved in a specific cellular response, and thereby couple a single phosphotyrosine site to several downstream targets.

Transcription factors

A direct route from cell-surface receptors to the regulation of gene expression involves the activation of latent transcriptional regulators at the plasma membrane, as observed for the STAT proteins. STATs are SH2-containing transcription factors that also possess DNA-binding and transcriptional activation domains. STATs are recruited through their SH2 domains to phosphotyrosine sites on cytokine receptors and consequently are themselves phosphorylated[56,57]. Tyrosine phosphorylation elicits a mutual SH2–phosphotyrosine interaction between two STAT chains, resulting in dimerization and relocalization of the STAT oligomer to the nucleus, where it regulates gene expression.

Regulators

Mammalian cells have a limited number of core signaling pathways, which are used repeatedly to achieve a remarkable diversity of biological responses. Thus, specificity in signaling probably depends not only on the selection of a particular pathway but also on the kinetics with which it is activated and the expression of adaptors and targets (i.e. transcription factors) in the relevant cell. Regulatory proteins might therefore be crucial in molding a generic pathway into a specific response. A number of SH2 proteins have regulatory functions. c-Cbl has an N-terminal SH2 domain, which is part of a larger folded structure that includes a four-helix bundle and an EF-hand linked to a RING-H2 domain that binds to E2 protein–ubiquitin ligases[58]. Binding of the c-Cbl SH2 domain to activated RTKs can therefore induce their ubiquitination and downregulation[59].

STAT-mediated cytokine signaling stimulates the expression of SH2-containing proteins that have a C-terminal SOCS box capable of binding to the elongin B/C complex, also implicated in protein ubiquitination[60]. Such SOCS proteins repress STAT signaling, potentially both by binding and directly inhibiting the receptor-associated JAK tyrosine kinases and by stimulating ubiquitination. In addition, the SOCS-3 protein might enhance Ras signaling by binding to and inhibiting p120-RasGAP, suggesting that inducibly expressed SOCS proteins might redirect signaling pathways downstream of cytokine receptors[61].

Although SH2 domains are usually found in the context of other modules, the SH2D1A protein (also termed SAP) is comprised almost exclusively of a single SH2 domain, which is unusual in binding stably to motifs with the consensus Thr-Ile-X-Tyr-X-X-Val, even in their unphosphorylated state[62–64]. SH2D1A binds to such a motif in the cytoplasmic tail of the CD150/SLAM receptor on T cells in a phospho-independent fashion, although the affinity of the interaction is increased about fivefold by phosphorylation. Structural and mutagenesis data indicate that the SH2D1A SH2 domain has a more extensive peptide-binding surface than other SH2 domains and recognizes specific residues both N- and C-terminal to the central tyrosine, accounting for its ability to bind to unphosphorylated motifs. The physiological importance of these interactions is suggested by the finding that the SH2D1A SH2 domain is mutated in patients with X-linked lymphoproliferative syndrome (XLP), a disease that renders these individuals extremely sensitive to the effects of Epstein–Barr virus infection, which results in a fatal infectious mononucleosis, as well as dys-gammaglobulinemia and malignant lymphoma[62]. Disease-causing mutations in the SH2D1A SH2 domain that compromise peptide recognition usually result in death by the age of 40 in humans. Although not essential for lymphoid development, and therefore not likely to be part of the core lymphoid signaling machinery, SH2D1A plays an important modulatory role in T cells and, potentially, natural killer (NK) cells as XLP disease results from abnormal lymphoid responses. SH2D1A might have a suppressive function in T-cell signaling, either by blocking access of positive regulators such as the Shp2 phosphatase to the SLAM receptor[62] or by recruiting tyrosine kinases to phosphorylate sites in SLAM that subsequently bind to negative regulators such as the SH2 5′-inositol phosphatase SHIP (Ref. 65).

Evolution of signaling networks

A number of conclusions can be drawn from these findings that are of general relevance for protein-interaction modules. First, SH2 domains are linked to a wide variety of catalytic and interaction domains, and the functions of SH2-containing proteins are therefore built up in a combinatorial fashion by the joining of multiple, independently folding modules that direct the protein to an appropriate subcellular location and control its interactions with receptors and intracellular targets. It seems probable that the SH2 domain evolved coordinately with tyrosine kinases in early metazoan animals as a way of coupling phosphotyrosine signals to cytoplasmic targets. By joining an SH2 domain to a pre-existing catalytic or interaction domain, a novel link from phosphotyrosine to an intracellular signaling pathway could be rapidly created. Thus, the cassette-like construction of eukaryotic signaling proteins, and the repeated use of conserved interaction domains, might have allowed the rapid formation of novel pathways and the evolution of increasingly complex signaling networks to control the cellular response to external signals.

Another point is that SH2 domains can be flexible in their binding properties, as shown by the ability of the SH2D1A SH2 domain to recognize unphosphorylated peptides. Similarly, some PTB domains bind only to unphosphorylated sites and might originally have evolved for peptide binding and only subsequently achieved phosphotyrosine recognition[17]. Indeed, PTB domains belong to a larger family that includes PH domains (which recognize phosphoinositides), EVH1 domains (which bind to proline-rich sequences) and subdomain 3 of FERM domains (involved in membrane anchoring). Although these have quite distinct ligand-binding properties and amino acid sequences, they have a conserved structural fold that

serves as a flexible scaffold that can be modified to accommodate numerous intermolecular interactions[17]. Conversely, although they have entirely different structures, PH, FYVE, PX, ENTH and Tubby domains all bind to specific phosphoinositides[66], while the distinct SH3, WW and EVH1 domains recognize proline-rich motifs that adopt a polyproline type II helix[67].

Regulation of modular protein- protein interactions by posttranslational modifications - a general concept

The role of protein phosphorylation in mediating protein–protein interactions was discovered in the context of tyrosine kinase signaling, but there are a growing number of domains, akin to the SH2 and PTB domains, that bind to specific phosphoserine/threonine motifs. The dimeric 14–3–3 proteins bind to phosphoserine-containing sequences in a wide range of proteins[68,69]. Such interactions regulate protein localization, as in the case of the Cdc25c phosphatase, which dephosphorylates and activates the cell-cycle[73] kinase Cdc2. Phosphorylation of Cdc25c during interphase, or in response to DNA damage, results in 14–3–3 binding and consequent retention of Cdc25c in the cytoplasm where it cannot gain access to Cdc2[70]. 14–3–3 binding can also induce a conformational change in associated enzymes, such as serotonin *N*-acetyl transferase, resulting in regulation of catalytic activity[71].

Serine/threonine phosphorylation is also crucial in marking specific proteins for recognition by SCF-type E3 protein–ubiquitin ligases. The substrate-recognition subunits of SCF complexes contain an F-box, which anchors them to the complex, and a variable C-terminal region that binds to the target for ubiquitination[72]. F-box proteins such as yeast Cdc4 and mammalian βTRCP have a C-terminal WD40 repeat domain that binds to serine/threonine-phosphorylated targets. Cdc4, for example, recognizes phosphorylated Sic1, an inhibitor of the S-phase cyclin-dependent kinase, and, by inducing Sic1 ubiquitination and degradation, controls passage from G1 to S phase in the cell cycle[73]. Similarly, βTRCP recognizes the form of β-catenin phosphorylated by GSK3β, inducing β-catenin destruction and blocking adventitious signaling through the Wnt pathway[74]. Mutations in β-catenin that block this phospho-dependent interaction are associated with colon cancer since they cause constitutive activation of the β-catenin pathway.

FHA domains recognize phosphothreonine-containing motifs (i.e. pThr-X-X-Asp) and are found in a wide range of proteins involved in events such as DNA repair and protein trafficking[75]. In the best-studied example, the N-terminal FHA domain of the yeast protein kinase Rad53 binds to phosphorylated sites on the protein Rad9, and this binding is crucial for the response to DNA damage[76].

These latter observations emphasize that signaling events in the nucleus are controlled in ways similar to those in the cytoplasm. This is exemplified by the finding that bromodomains, a common feature of histone acetyl transferases (HATs), bind to specific acetylated lysine motifs in histones, in a manner that contributes to their biological activity[77]. In the same way that phosphorylation of tyrosine or threonine residues creates a binding site for SH2 or FHA domains, acetyl-lysine residues protrude into a deep and conserved cleft in bromodomains[78], which are thereby recruited along with their linked HAT modules to chromatin. By contrast, the chromo domains of proteins such as HP1 bind to histone motifs with methylated lysines and mediate gene silencing[79]. These findings argue that posttranslational modifications other than phosphorylation direct the formation of protein complexes through the recruitment of interaction domains.

The future

The completion of the genome sequences of several metazoan organisms, including human, provides a comprehensive inventory of interaction domains and signaling enzymes. Using proteomic techniques, such as mass spectrometry, the yeast two-hybrid system, phage display and peptide library screening, together with predictive bioinformatic approaches, it might be possible to establish a comprehensive picture of the wiring circuitry of human cells and of the aberrations in this interaction network that accompany disease states. Indeed, based on the idea that much of cellular organization can be described in terms of 'molecule A' binds to 'molecule B', we have developed a bioinformatic tool (BIND) to describe and analyze the large amounts of interaction data currently being generated and to simulate cellular behavior[80]. Having arrived at a simplifying principle in understanding cellular organization, based on the reiterated use of interaction domains and motifs, a challenge for the future is to develop computational methods to allow us to assimilate interaction data and to determine how simple molecular interactions can be employed to build a cell and a living organism.

Acknowledgements
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Minireview

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Increasing Complexity of the Ras Signaling Pathway*

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Ras is a key regulator of cell growth in all eukaryotic cells. Genetic, biochemical, and molecular studies in *Caenorhabditis elegans*, *Drosophila*, and mammalian cells have positioned Ras centrally in signal transduction pathways that respond to diverse extracellular stimuli, including peptide growth factors, cytokines, and hormones. The biological activity of Ras is controlled by a regulated GDP/GTP cycle. Guanine nucleotide exchange factors (GEFs[1]; RasGRF1/2 and Sos1/2) promote the formation of the active, GTP-bound form of Ras (1). GTPase-activating proteins (GAPs; p120 GAP and NF1) accelerate the intrinsic GTP hydrolytic activity of Ras to promote formation of the inactive, GDP-bound form of Ras (1). Mutations in Ras at amino acids 12, 13, or 61 make Ras insensitive to GAP action and, hence, constitutively active in transforming mammalian cells (2, 3). These activating mutations in Ras are prevalent in a wide spectrum of human cancers. It has been estimated that 30% of all human tumors contain an activating mutation in Ras. The frequency of Ras mutations varies depending on tumor type, with the highest frequencies seen in lung, colon, thyroid, and pancreatic carcinomas (3). The frequency of Ras mutations is likely to be an underestimation of the contribution of aberrant signaling through the Ras pathway to human malignancies because chronic up-regulation of the Ras pathway can occur in the absence of mutations in Ras itself (4–6).

Ras Directly Binds Raf and Activates a Kinase Cascade

Ras mediates its effects on cellular proliferation in part by activation of a cascade of kinases: Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK kinases 1 and 2), and ERK1/2 (7). Upon activation, the ERKs phosphorylate cytoplasmic targets (such as Rsk (8) and Mnk (9, 10)) and translocate to the nucleus, where they stimulate the activity of various transcription factors that include the Elk-1 transcription factor (Fig. 1). Ras activates this kinase cascade by directly binding to Raf (11, 12). The binding of Ras to Raf requires active, GTP-bound Ras and an intact effector domain. The recent observation that Ras interacts with two distinct NH_2-terminal regions of Raf-1 (RID/RBS1, spanning residues 51–131 (13, 14) and Raf-CRD (14)) suggests that Ras promotes more than just membrane translocation of Raf and instead may also facilitate the subsequent events that lead to Raf-1 activation. Other components that contribute to Raf-1 activation include 14-3-3 proteins, phospholipids, and serine/threonine and tyrosine kinases (15). Therefore, the connection between Ras and Raf alone is not simply linear and requires multicomplex formation to complete Raf activation.

Ras Targets Multiple Effectors

Ras is likely to act through additional proteins besides Raf. The earliest observations that Ras has multiple effector proteins came from genetic studies in the budding yeast *Saccharomyces cerevisiae* and later the fission yeast *Schizosaccharomyces pombe*. Budding yeast devoid of Ras function were inviable, but yeast lacking adenylyl cyclase, an effector of Ras in this organism, were often capable of forming slow growing microcolonies (16). This result suggested that Ras proteins in *S. cerevisiae* have an essential function other than the activation of adenylyl cyclase. In *S. pombe*, Ras directly interacts with two effectors: Byr2, a MAPK kinase kinase, and Scd1, a GEF for the Rho family protein Cdc42 (17). Three additional observations in mammalian cells indicated that the events downstream of Ras are more complex than simply activating the Raf kinase. First, activated Raf induces only a subset of the events mediated by activated Ras. For example, activated Ras activates three distinct MAPK cascades (ERK, JNK, p38), whereas Raf causes direct activation only of ERK (18, 19). Second, activated Raf is not sufficient to promote all functions of Ras, such as the transformation of some epithelial cells (20). Third, studies with Ras mutants that discriminate between effectors suggest that multiple effector-mediated pathways are important for establishing and maintaining the transformed state (21, 48).

A plethora of candidate Ras effectors in addition to Raf have been reported. These include p120 Ras GAP (22), GEFs for the small GTPase Ral (RalGDS, RGL, RLF/RGL2) (23), AF6/Canoe (24, 25), RIN1 (26), and phosphatidylinositol 3-kinase (PI3K) (27). Although these candidate effectors comprise a very diverse collection of structurally and functionally distinct proteins, they all show preferential affinity for active Ras-GTP. Therefore, it is not surprising that residues corresponding to the switch I (Ras residues 30–37) and II (residues 59–76), which define the conformation differences between the GDP- and GTP-bound Ras, are involved in effector recognition. Specifically, an intact core Ras effector domain (residues 32–40) is essential for all effector interactions. Mutation of residues in sequences flanking this region (spanning residues 25–45) show differential impairment of effector interactions and provide useful mutants to decipher the contribution of specific effectors for Ras function (28). Thus, Ras residues important for effector interaction are more extensive than originally believed. The interaction of Ras with candidate effectors is often direct (interaction is observed *in vitro* using proteins purified from bacteria). For some, the interaction with Ras is observed *in vivo* upon co-immunoprecipitation, but these experiments are often done under conditions in which the Ras target is overexpressed. To date, Raf is the only Ras target protein for which genetic studies confirm its fundamental role in Ras signaling in a normal cellular context. Nonetheless, the interaction of Ras with at least some of these target proteins is likely to be critical

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; ERK, extracellular receptor-stimulated kinase; MEK, MAPK/ERK kinase; RID, Ras-interaction domain; RBS, Ras-binding site; CRD, cysteine-rich domain; JNK, Jun NH_2-terminal kinase; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; CAAX, cysteine, aliphatic, aliphatic, terminal amino acid.

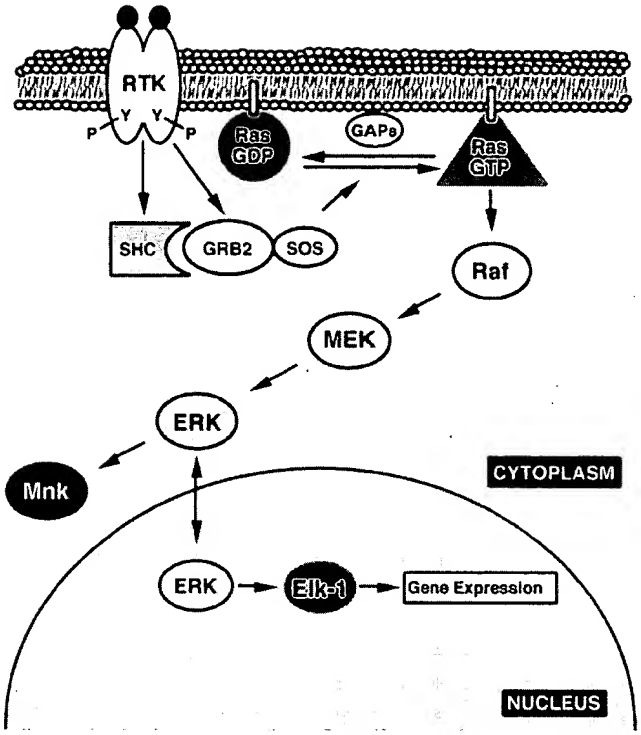

FIG. 1. **Ras regulates a cascade of kinases.** Ras is a GDP/GTP-regulated binary switch that resides at the inner surface of the plasma membrane and acts to relay extracellular ligand-stimulated signals to cytoplasmic signaling cascades. A linear pathway where Ras functions downstream of receptor tyrosine kinases (RTK) and upstream of a cascade of serine/threonine kinases (Raf > MEK > ERK) provides a complete link between the cell surface and the nucleus. Activated ERKs can translocate into the nucleus to phosphorylate and activate transcription factors, such as Elk-1. Activated ERKs also phosphorylate substrates in the cytoplasm, including the Mnk kinase, and thus contribute to translation initiation of mRNAs with structured 5′-untranslated regions.

for mediating the role of oncogenic Ras in malignant transformation.

Multiple Effector Pathways Contribute to Ras-mediated Transformation

What is the contribution of each of the known effector-mediated pathways to malignant transformation? The current state of affairs is depicted in Fig. 2. As described earlier, activation of the Raf/ERK pathway, with its concomitant activation of transcription factors, is essential for cell proliferation. The Ras GTPase-activating protein, p120 GAP, in addition to negatively regulating Ras function may impinge on the Rho family via its association with p190, a GAP for Rho family members (29). Activation of members of the Rho family of GTPases is likely to contribute significantly to the Ras-transformed phenotype (reviewed in Ref. 30).

The family of GEFs for Ral have also been implicated as target proteins for Ras (31, 32). A role for Ral in regulation of phospholipase D and in actin cytoskeletal rearrangements (via interaction with RalBP1) has been suggested (33, 34). In one report, RalA has been reported to cooperate with Ras for transformation (35), but others have not seen this cooperativity (36). Perhaps the RalGDS targets other proteins in addition to Ral that can influence the transformed phenotype. There is precedence for multiple functions residing in GEFs; SOS facilitates the exchange of nucleotides on Ras and couples Ras to Rac through its Dbl and pleckstrin homology (PH) domains in a PI3K-dependent manner (37).

RIN1 was identified in a genetic selection for mammalian cDNAs that were capable of suppressing the phenotypes associated with constitutive activation of the Ras pathway in *S. cerevisiae* (38). RIN1 interacts directly with Ras in a GTP- and effector domain-dependent fashion and localizes to the plasma membrane (26). Subsequently, RIN1 was shown to interact with Abl and Bcr/Abl *in vitro* and *in vivo* through a domain distinct from the Ras binding domain (39, 40). Moreover, RIN1 can enhance the transforming activity of Bcr/Abl and rescue several transformation-defective mutants of Bcr/Abl (40). The aspects of Ras function mediated by RIN1 are still the subject of investigation, but one possibility is that RIN1 coordinates signals from Ras and Abl.

Biochemically, AF6/Canoe are candidate Ras effectors (25). In addition, genetic studies in *Drosophila* have linked Canoe to Ras in eye development (41). Canoe/AF6 have a GLG(F/D)HR motif, a conserved sequence found in proteins that associate with cellular junctions, so perhaps Canoe/AF6 coordinate signaling events at the plasma membrane to remodeling of the actin cytoskeleton.

Finally, activation of PI3K, via a direct interaction between Ras and the catalytic subunit of the protein, is necessary for actin cytoskeletal rearrangements associated with the transformed phenotype (36). PI3K is a lipid kinase with specificity for the 3-position of the inositol ring. Activation of PI3K by a variety of extracellular stimuli leads to the accumulation of the second messenger phosphatidylinositol 3,4,5-trisphosphate. What are the downstream targets of this second messenger? One target is the serine/threonine kinase Akt/PKB. Binding of Akt/PKB via its PH domain to phosphatidylinositol 3,4,5-trisphosphate localizes Akt/PKB to the plasma membrane and leads to a partial activation of its kinase activity (42). Akt/PKB activity is further increased by phosphorylation on 2 residues by two different kinases, one of which, PDK1, is itself a lipid-regulated kinase (43). The events downstream of Akt/PKB are the subject of intense investigation in many laboratories. Akt/PKB phosphorylates and inactivates the pro-apoptotic protein BAD (44, 45) but is likely to have additional substrates. Other targets of the products of PI3K include the PH domains of Vav (46), SOS (37), and GRP1 (47).

One of the more elegant approaches to understanding the contribution of each of the effector pathways to Ras-mediated transformation has been the use of Ras effector mutants that are impaired in binding a specific target (21, 36, 48, 49). For example, studies with effector domain mutants have revealed a bifurcation of the signaling pathways downstream of Ras leading to remodeling of the actin cytoskeleton and DNA synthesis. RasV12C40, an activated mutant of Ras with an alteration of tyrosine to cysteine at position 40 in the effector domain, is unable to bind Raf. This mutant fails to activate the ERK cascade and cannot activate a Ras-responsive reporter construct, but it is capable of inducing membrane ruffling to the same extent as an activated Ras with an intact effector domain (49). These results suggest that stimulation of membrane ruffling and activation of the ERK cascade are mediated by distinct Ras effector proteins. Subsequently, RasV12C40 was shown to bind to and activate PI3K, suggesting that Ras-induced morphological alterations may be mediated in part through activation of PI3K (36). In addition to binding PI3K, RasV12C40 will also interact with AF6 (48), and a role for AF6 in modulation of the actin cytoskeleton by RasV12C40 cannot be excluded. Finally, the ability of this mutant to cause tumorigenic transformation demonstrates that Raf-independent pathways alone are sufficient to promote Ras transformation.

Two additional approaches to dissect the contributions of this surfeit of candidate effector proteins to Ras function have been to overexpress or membrane-target a specific effector to

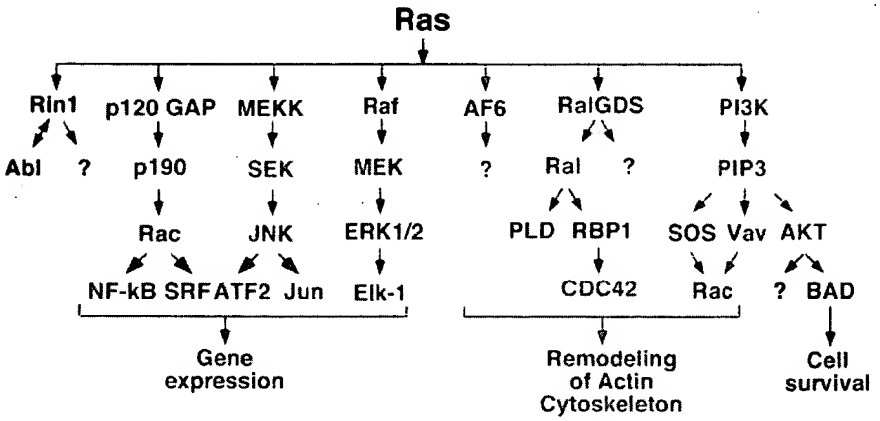

FIG. 2. **A surfeit of candidate Ras effectors.** Multiple effector pathways contribute to Ras function. Our current understanding of the downstream targets of each of the Ras effectors is shown in the figure (see text for details). *PLD*, phospholipase D; *PIP3*, phosphatidylinositol trisphosphate; *MEKK*, MEK kinase; *SEK*, SAPK/JNK kinase; SRF, serum response factor.

see if this mimics any aspect of Ras function. Targeting to the plasma membrane is often achieved by adding the sequence containing the CAAX box of Ras to the effector of interest. Membrane targeting has been shown to cause constitutive activation of Raf and PI3K. If membrane targeting of the candidate effector does not reproduce any aspect of Ras function, it may be that the target protein under study is already constitutively localized in this cellular compartment. Although Raf and PI3K reside in the cytoplasm and become associated with the plasma membrane upon receipt of stimulatory signal(s), other Ras target proteins, such as adenylyl cyclase in yeast and the RalGDS, are constitutively membrane localized. Membrane-targeted RalGDS did not exhibit any transforming potential.[2] Finally, a powerful (but as yet not common) approach to decipher the role of a Ras target protein is to determine whether fibroblasts derived from mice deficient in a target protein are impaired in transformation (for example, see Ref. 50).

Ras Mediates Life and Death Decisions by Distinct Effector Pathways

One perplexing aspect of the Ras signaling pathway is that Ras can promote both cell death and cell survival through interactions with distinct effector proteins. Using Ras mutants, Kauffmann-Zeh *et al.* (51) demonstrated that activation of Raf by Ras promotes apoptosis in fibroblasts containing an inducible c-Myc oncoprotein, whereas activation of PI3K by Ras promotes cell survival. In this assay, oncogenic Ras enhanced apoptosis. This result suggests that Ras has the potential to trigger two seemingly contradictory biological outcomes: cell death by activation of Raf and cell survival by activation of PI3K. At least in this assay system, cell death (the Raf pathway) is dominant over cell survival (the PI3K pathway). How this seemingly discordant choice of cell death *versus* survival is achieved is not known.

What is the contribution of each of these pathways to tumor initiation and/or progression? Promotion of cell death by activation of Raf may be an important factor in limiting the expansion of cells harboring Ras mutations, whereas promotion of cell survival by activation of PI3K may contribute to tumor expansion and metastases. It will be interesting to see if the relative contributions of these antagonistic pathways can be modulated by other signaling pathways and whether such modulation plays a role in tumor formation.

Activated Ras also promotes cell survival in epithelial cells upon detachment from an extracellular matrix (52). This action of Ras is mediated through activation of PI3K and Akt/PKB. Given that the majority of human tumors are of epithelial cell origin, a pharmacological intervention that could switch a Ras-dependent survival signal into an apoptotic signal might be of considerable value in the treatment of human malignancies.

[2] G. J. Clark and C. J. Der, unpublished observation.

Summary

The last 5 years have seen an impressive expansion in the number of candidate Ras effectors. Much progress has been made toward deciphering the aspects of Ras function mediated by each of these proteins, and many studies, in particular those with effector domain mutants, have convincingly demonstrated that Ras must target at least three different pathways for transformation. The corruption of the signaling pathways that lie downstream of Ras is a recurring theme in the initiation and/or progression of human malignancies. Pharmacological interventions have directly impeded Ras function by interfering with its farnesylation and membrane targeting or have blocked activation of components of the kinase cascade downstream of Ras (53). However, the function of Ras and its downstream kinase cascade is central to many cellular processes, and this may limit the usefulness of these approaches. The diversity of Ras target proteins and the necessity for activation of multiple effector pathways for malignant transformation by Ras open new directions for the design of additional therapeutic interventions that may negate Ras transformation without abolishing all of Ras function.

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